Free Radical Damage and Noise-Induced Hearing Loss: *in vivo in situ* Sensing

Final Report, July 2008

List of Illustrations

Statement of Problem

Noise-induced hearing loss (NIHL) is one of the most pervasive sources of reduced auditory acuity, the biochemistry of which is incompletely understood. We hope to clarify the role of reactive oxygen species (ROS: singlet oxygen $^1O_2$, hydrogen peroxide H_2O_2, superoxide ion $O_2^{-\bullet}$, hydroperoxyl radical $HO_2^{\bullet}$,and hydroxy radical $OH^{\bullet}$) frequently associated with NIHL, and in the process demonstrate technology generally applicable to studying small-molecule chemistry in the inner ear. Inner ear structures (cochlea) show evidence of damage by ROS correlated with NIHL. It is unclear whether this damage CAUSES hearing loss or is a CONSEQUENCE of the processes leading to such loss. Our goal has been to develop, validate, and employ unique chemical sensors, implanted in laboratory animal cochlea, to measure ROS in real time. This may lead to development of strategies to optimize pharmaceutical intervention to minimize NIHL.

While NIHL is a liability in the general population, it is both an acute and chronic problem for soldiers and veterans.[1-3] While there has been much excitement that hair cell regeneration may be stimulated in mammals,[4, 5] it is unlikely that this will be clinically important for some time, if ever. It has been shown[6] that NIHL, neural (but not conductive) hearing loss with age, and ototoxicity by some antibiotics[7] share some metabolic features, specifically that their effect can be reduced by the use of anti-oxidants and radical scavengers. In the last decade (but, in a few cases, earlier[8]), it has become evident that oxidized metabolites[9, 10] and stable oxidants (particularly NO[11-13]) are correlated with NIHL. Pharmacological protection from NIHL by anti-oxidants gives ambiguous outcomes.[14-21] While some drugs are efficacious, elevation of superoxide dismutase levels fails to protect against NIHL.[22]

The critical issue in the link between ROS and NIHL is the difference between *correlation* and *causation*. What are the primary or first-appearing ROS? How, when, and where within the inner ear are they produced? Do they arise after hair cells and other inner ear structures are damaged by intense noise, or are they produced in the lumen of the cochlear duct and *scalae* by that noise (through, e.g. imploding, locally nucleating bubbles[23-26]), leading to cellular damage? While ROS can kill cells, they are also part of the apoptotic cascade. Is the useful role of anti-oxidants in protecting hearing from NIHL to suppress apoptosis or to prevent formation of the species initiating apoptosis?[18, 27] Other hypotheses for ways that intense noise could damage cells include, e.g., intense noise leading to overstimulation of hair cells and subsequent abnormal ion fluxes, with loss of homeostasis and cell rupture due to imbalanced osmotic pressure. In this case, ROS would appear when mitochondria, or other heme-enzyme-containing structures, spill into the cochlear duct and form ROS from dissolved oxygen.[28] One might invent other scenarios, but the missing link is chemically-specific information gathered WITHIN the cochlea DURING noise exposure, as opposed to current methods that sense consequences of exposure *post facto*. Implantable, species-selective sensors with good spatio-temporal resolution are the needed tools.

Our purpose here is to develop microsensors specific for $O_2^{-\bullet}$, to demonstrate their stability, and then to collect preliminary *in vivo* data on $O_2^{-\bullet}$ formation in the cochlea of Mongolian gerbils.

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Summary of Most Important Results

Progress has been made in two areas: A) Development of Superoxide Sensing Electrodes and B) Development of a Microfluidic Superoxide Source. The latter was not part of our original goals, but has been found necessary so that the sensors may be reliably calibrated.

A) Development of Superoxide Sensing Electrodes

A CAD drawing of our microamperometric sensor is shown in Figure 1.

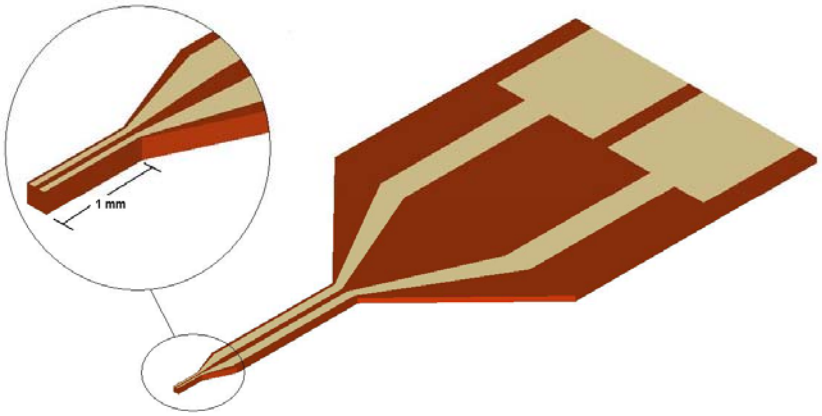

Figure 1. Microamperometric Sensor. Working electrodes shown; counter and reference electrodes are on the reverse side.

Electrodes are gold on Kapton® substrates. Reference electrodes (Ir/Ir_xO_y) are electrodeposited from $IrCl_3$ solution. Working electrodes are formed using 3-mercaptopropionic acid on which superoxide dismutase is adsorbed in the manner of Tian *et al.*[29, 30] For much of our work, sensing was performed with a CH-660 (CH Instruments, Austin, TX) cybernetic potentiostat. We have also used these electrodes, driven by the CH-660, but with a Keithley 6485 picoammeter for current to voltage conversion. The latter provided superior noise and dynamic range performance.

When calibrating the electrodes, we typically have used EDTA to sequester metal ions and prevent Fenton chemistry. While the mechanisms and rate constants of Fenton reactions are not still debated,[31, 32] the essential issue is common knowledge:

$$M^{n+} + H^+ + H_2O_2 \rightarrow OH^\bullet + H_2O + M^{+(n+1)}$$
$$M^{+(n+1)} + H_2O_2 \rightarrow M^{n+} + H^+ + HO_2^\bullet$$

Transition metals (M^{k+} in the equations) can dismutate H_2O_2 into radicals which, in turn, can participate further in chain reactions. Sequestration of metal ions with EDTA suppresses Fenton chemistry (the Haber-Weiss cycle), and thus acts to suppress radical chain reactions. Further, some metal ions can act as superoxide dismutases by analogous reactions. It was thus natural to add EDTA to buffers when attempting to characterize electrodes.

Superoxide generation was by reaction of O_2 with a reductant (either acetaldehyde or xanthine), catalyzed by xanthine oxidase. The rate of superoxide production was inferred from the slope of the absorbance vs. time curve at 550 nm, using a molar absorptivity change between ferri- and ferro-Cytochrome C of 2.12×10^4 M^{-1} cm^{-1} [33] and presuming reaction of Cytochrome C with superoxide was sufficiently rapid that dismutation was irrelevant. We further presumed that the rate of superoxide production did not diminish over a period of 2 hours, during which time a suicide reaction of xanthine oxidase can lead it to generate H_2O_2 directly rather than via $O_2^{-\bullet}$.[34-36] Thus, it is conceivable that actual superoxide production was below what we expected. Separate experiments demonstrated that at least

some $O_2^{-\bullet}$ was generated at least 3 hours after xanthine oxidase was suspended in buffer, so complete cessation of $O_2^{-\bullet}$ production during these experiments is inconsistent with observation.

Our initial attempt to repeat the Tian work resulted in the time series shown in Figure 2 (which appeared in our proposal for the current work). A sensitivity of 0.4 nA μM^{-1} on an electrode with geometric area 8000 μm^2 was obtained. This expected result has never been repeated by us. While numerous problems including electrical noise, grounding, signal amplification levels, and signal averaging were addressed, the lack of signal persisted over many experiments. In light of the irreproducibility of Figure 2, we decided to examine the state of Cu/Zn SOD on the electrode surface using cyclic voltammetry to reveal changes in redox behavior.

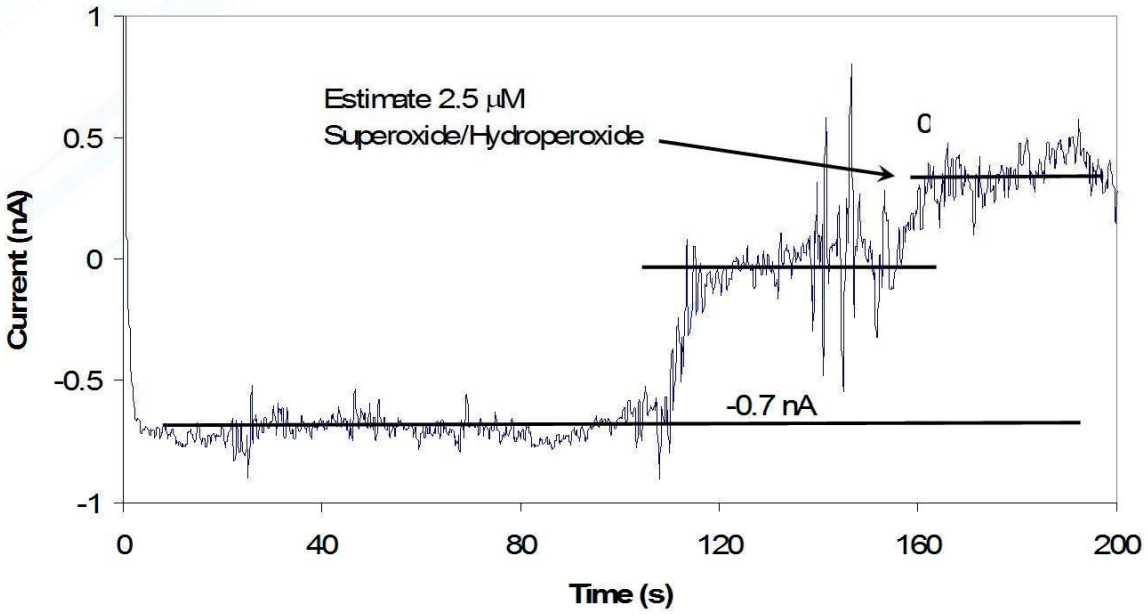

Figure 2. Response of Superoxide Dismutase Sensor to $O_2^{-\bullet}/HO_2^{\bullet}$ Produced with Xanthine Oxidase and Acetaldehyde. Xanthine oxidase solution mixed stepwise (~ 100 s and ~ 160 s) with buffer in which electrode was suspended.

Figure 3 shows the cyclic voltammogram of a 3-mercaptopropionic acid-coated gold electrode on which Cu/Zn SOD has been adsorbed. Immediately following adsorption, one sees characteristic peaks at ±0.15 V vs. Ir/Ir_xO_y at pH 7.4. Figure 3 also shows the disappearance of these peaks after the electrode has been exposed to a solution containing EDTA. The post-exposure voltammogram lacks the peaks characteristic of Cu oxidation and reduction. Additionally, current increases at cathodic potential, indicating more rapid production of H_2 than occurred prior to the introduction of EDTA. It is unlikely that SOD desorption can explain these data. At potentials less than 0.2 V, current is nearly unchanged in the presence of EDTA. The thiol layer is apparently intact, while the redox centers have disappeared.

In contrast, scavenging of metals from SOD by EDTA is consistent with the data in Figure 3. Table 1 shows the binding constants for SOD and EDTA with Cu and Zn insofar as these have been reported in the literature. As there are two binding sites for each cation, the competitive equilibria are:

$$2\ Cu^{2+} + 2\ Zn^{2+} + \text{apo-SOD} \rightarrow \text{active enzyme}$$

vs

$$Cu^{2+} + EDTA^{4-} \rightarrow CuEDTA^{2-}$$
$$Zn^{2+} + EDTA^{4-} \rightarrow ZnEDTA^{2-}$$

As noted in the Table, separate binding constants and pH dependence have been reported for the two copper sites, but not for the two zinc sites.

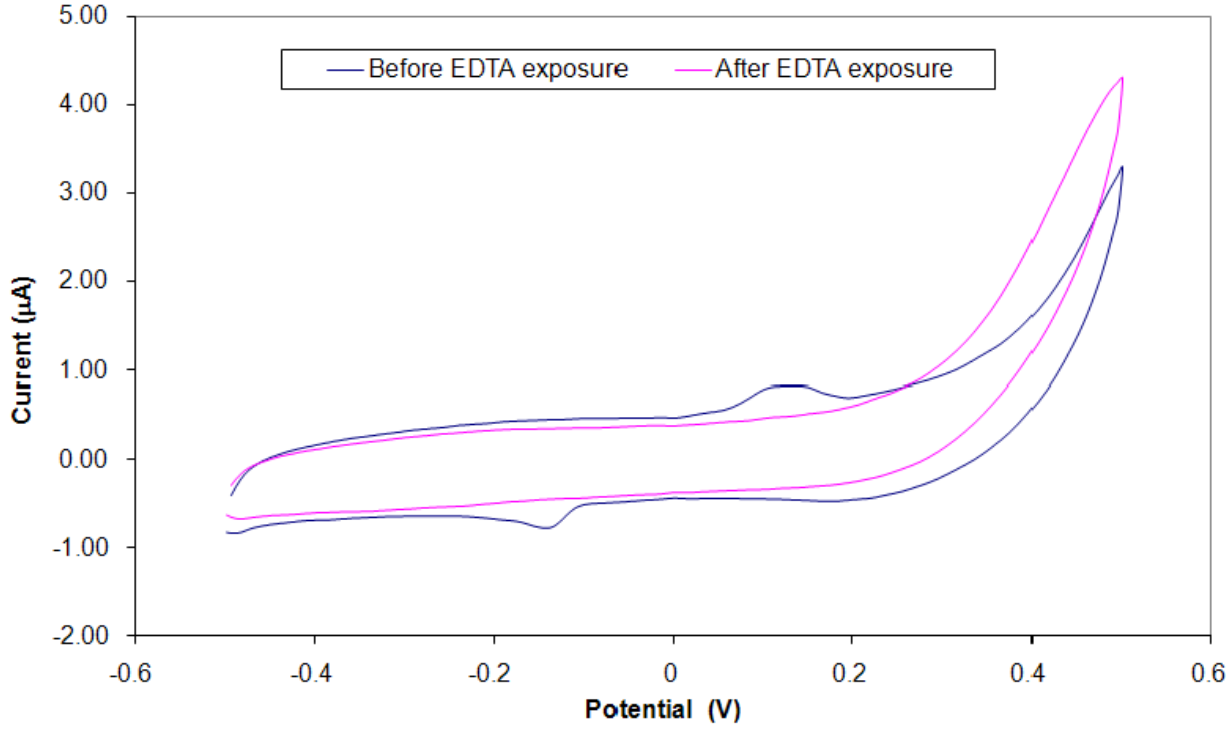

Figure 3. Cyclic Voltammetry of Superoxide Dismutase on Gold Electrode as Influenced by 0.1 mM EDTA.

Table I. Binding constants for SOD and EDTA with Cu^{2+} and Zn^{2+}.[37-39]

pH	K_f Cu^{2+}/SOD	K_f Zn^{2+}/SOD	K_f Cu^{2+}/EDTA	K_f Zn^{2+}/EDTA
6.25	$10^{13.9}$, $10^{13.4}$	$10^{11.1\pm0.1}$	$10^{14.4}$	$10^{12.1}$
7.4	10^{16}, $10^{14.5}$	?	$10^{15.8}$	$10^{13.5}$

Clearly, the binding constant for EDTA with Cu^{2+} is nearly the same as for SOD with the first Cu^{2+} and greater than with the second. Binding of EDTA with Zn^{2+} is similarly competitive. Thus, in an attempt to prevent artifactual superoxide dismutation in solution, we created conditions certain to destroy enzyme adsorbed on the thiol-coated gold electrodes. Adding millimolar Cu^{2+} and Zn^{2+} to solution and omitting EDTA preserved the form of the EDTA-free cyclic voltammogram. As we were about to test our electrodes for superoxide sensing in EDTA-free solutions, we became aware of the work of Chen *et al.*[40]. We have since replicated their sensing of superoxide using Cytochrome C as the active surface protein, and have also replicated our own earlier work as shown in Figure 1, in which Cu/Zn SOD electrocatalyzes interaction of a gold electrode with $O_2^{-\bullet}$. While encouraging, these observations point to several considerations in developing and using metalloprotein-based sensors:

- Metalloprotein integrity may require that the sensing layer be protected by an anion exchange membrane. Any trafficking in cations risks eventual depletion of the protein's metallic cations.

- Such overlayers will slow electrode response compared to diffusion limit in solution.
- Optical detection has no advantage over electrochemical detection; optical shifts due to interaction of metalloproteins with radicals are just as subject to error from demetalization as are electrochemical measurements.
- *in vivo*, the high concentration of amino acids and polypeptides in addition to proteins means that sensor destruction by abstraction of metal ions from immobilized proteins must be expected. Anion exchange membranes may be key to protecting sensors.
- While one expects that anion exchange membranes will be non-reactive toward $O_2^{-\bullet}$, there is no general way to ensure that the membranes are inert towards $HO_2^{\bullet}$. At pH 7 and above, less than 1% of superoxide/hydroperoxide species are protonated at any moment, but reaction with hydroperoxide might gradually degrade the protective anion exchange membrane. We plan tests over an extended period to detect membrane degradation.
- The realization of diffusion-limited sensing of reactive oxygen species at biological pH remains elusive. Steady-state detection of radical flux remains considerably simpler and more reliable.

B) Development of a Microfluidic Superoxide Source

Among the reasons chelating agents have so prominently complicated sensor development is that no reservoir of aqueous $O_2^{-\bullet}$ is feasible at neutral pH due to the rate of spontaneous dismutation.[41] So that we can deliver controlled amounts of $O_2^{-\bullet}$ to electrodes without the complexity, irreproducibility, expense, and lack of portability of the xanthine/xanthine oxidase system, we are developing a microfluidic system that stores $O_2^{-\bullet}$ at pH>13, then (via ion-exchange) drops the pH to ~ 10, catalytically removes H_2O_2, then drops the pH farther to biological range (6.8 to 7.8). Figure 4 illustrates the idea, shows an exploded machine drawing, and a picture of the device, currently under test.

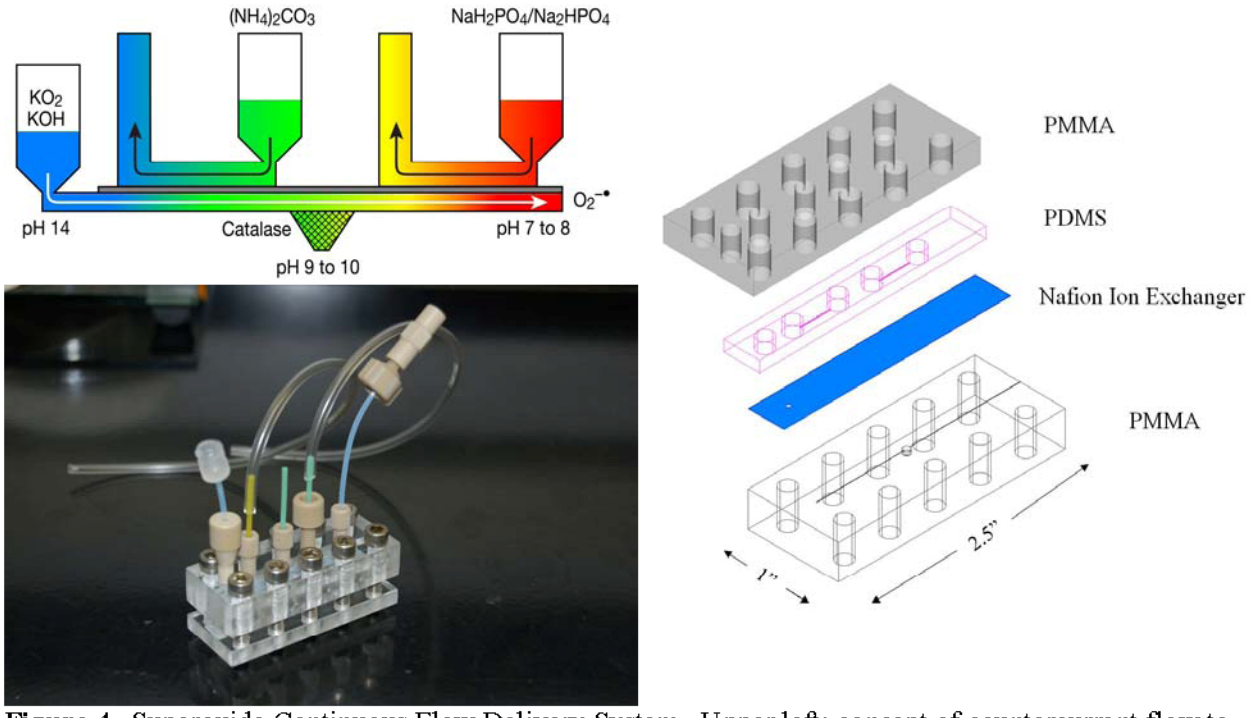

Figure 4. Superoxide Continuous Flow Delivery System. Upper left: concept of countercurrent flow to adjust stream pH by diffusively exchanging K^+ for H^+. Lower left: photograph of device currently being tested. Upper Right: Exploded machine drawing of flow system components.

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